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CULTIVATION OF AFRICAN AND SOUTH AMERICAN TRYPANOSOMES OF MEDICAL OR VETERINARY IMPORTANCE

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Today most parasite stages of the African trypanosome species can be grown in vitro. The co-cultivation of insect or mammalian cells is still necessary for the cultivation of some of the insect or mammalian forms. However, simple culture systems that produce high parasite yields cost-effectively have still to be found, with the exception of the procyclic trypomastigote forms which can easily be grown in semi-defined media. A culture system for the production of metacyclic forms in large numbers is a major goal for future research.

The different stages of *T. cruzi* can easily be grown in large quantities. Even metacyclic forms can be produced in large numbers in monophasic media without host cells. The vertebrate forms, grown in the presence of mammalian cells, can also be grown in quantities sufficient for most biochemical and immunological investigations. Either amastigotes or trypomastigotes can be obtained from cultures. They can be separated from host cells or debris by chromatography or gradient centrifugation.

The development of in-vitro culture systems has high priority in trypanosomiasis research. Progress achieved with African and South American trypanosomes opens new possibilities for various studies in vitro under controlled conditions. The field of applications is wide; the following list gives some idea of what can be attempted with trypanosome cultures:

1. Studies of metabolism and establishment of the nutritional requirements: for such studies, defined and serum-free media are of an enormous advantage. For other purposes, semi-defined or even complex media may be sufficient, besides being cheaper and resulting in higher parasite yields.
2. Screening of compounds to find new therapeutic drugs: testing for drug resistance without the interference of the host.
3. Production of antigens for serodiagnosis.
4. Mass production of specific parasite stages for biochemical and immunological investigations.
5. Study of differentiation processes: transformation steps are crucial phases in the parasite's life cycle. Blocking of such steps would stop further development of the parasite. This could be another method of control in addition to chemotherapy.
6. Replacement of the use of laboratory animals, or at least a reduction in their numbers. With some of the new animal protection laws enforced in European countries, scientists will be obliged to apply in-vitro culture methods. Cultures may also prove to be the ultimate way to grow a parasite for which there is no susceptible laboratory animal (e.g. bloodstream stages of *Trypanosoma b. gambiense*).
7. Production of insect stages in countries where handling the insect vector is not generally allowed (e.g. *Glossina* species in the USA).

This list does not claim to be complete, but it should serve to illustrate the wide range of applications and the advantages of trypanosome culture systems.

African Trypanosomes

Significant progress in the in-vitro cultivation of African trypanosomes has been made during the last eight years. Most research activity has been concentrated on the species *Trypanosoma* (*Trypanozoon*) *brucei* containing the human pathogenic subspecies *T.b. rhodesiense* and *T.b. gambiense*. But work has also enabled the development of culture systems for the animal infective subspecies *T.b. brucei*, as well as *T. congolense* and *T. vivax*. Today most of the parasite stages in the life cycle of the above-mentioned trypanosome species can be cultivated in vitro. However, with few exceptions, the culture systems are still inefficient in producing large numbers of parasites. Further research should concentrate on the development of cost-effective culture methods to grow the important stages of the parasite in mass cultures. In this paper only recent advances concerning trypanosome species which are of medical or veterinary importance are considered. For earlier work on the cultivation of African trypanosomes see review articles.[1–5]

Trypanosoma (*Trypanozoon*) *Brucei*

Procyclic trypomastigotes. The procyclic trypomastigote forms found in the midgut of the tsetse fly vector are non-infective for mammalian hosts and lack a surface coat. As early as the beginning of this century, this form of *T.(T.) brucei* could be grown in vitro. Today procyclic trypomastigotes can easily be produced in large quantities in complex,[5] semi-defined[6–8] or even in a defined medium.[9] This defined medium, however, only supports growth of selected stocks.

Cultures of procyclic forms are usually initiated with bloodstream forms from a mammalian host. The bloodstream forms, mainly the intermediate and stumpy forms,[10] normally transform within 48 h to forms morphologically very similar to procyclic trypomastigotes. The physiological transformation, however, proceeds more slowly and it takes about 4 weeks before an established culture is produced.

Some mainly monomorphic stocks which lack stumpy forms may be very difficult and slow to transform to procyclic trypomastigotes. A significant increase in the rate of transformation can be achieved by the addition of tricarboxylic acid cycle intermediates to the culture medium,[11] a method used routinely in our laboratory. Citrate and cis-aconitate in a 3 mM concentration have proved to be the two intermediates which are able to stimulate transformation. Another way to obtain a procyclic culture is the direct transfer of midgut forms from an infected Glossina into culture medium. This method is especially helpful with *T.b. gambiense* stocks which are very difficult to grow in sufficient numbers in laboratory animals. The tsetse vector may develop a midgut infection from a very low parasitaemia in a rodent host, whereas it is almost impossible to obtain enough bloodstream forms from such an animal for an in-vitro transformation.

Due to the absence of a surface coat, procyclic culture forms cannot replace bloodstream forms. However, there are some interesting applications of these cultures, e.g. the analysis of nuclear DNA, the demonstration of isoenzyme patterns for the characterization of stocks and the provision of Glossina infective forms. The infection of flies with procyclic trypomastigotes using membrane feeding has proved to be a useful tool in the cyclical transmission of *T.b. gambiense* stocks. To transmit a *T.b. gambiense* stock we use the following procedure. *Mastomys natalensis* are infected with a stabilate. A batch of tsetse flies is infected by feeding on these animals which normally show an extremely low parasitaemia. Some of the flies develop a midgut infection and these midgut forms are transformed directly into a procyclic culture medium. With the resulting procyclic culture forms, large numbers of tsetse flies can be infected using membrane feeding techniques.

Epimastigote forms. There is at present no culture system to grow the epimastigote form. In the tsetse fly, epimastigotes are not as dominant as the procyclic forms and are therefore often overlooked. Nevertheless, the epimastigote stage is of great importance for the development of the metacyclic forms in the salivary glands. The transformation of procyclic culture forms to epimastigotes is likely to be necessary for the production of metacyclic trypanosomes in vitro. In the salivary glands, the epimastigote forms attach to the gland epithelium; therefore, in order to grow epimastigotes in culture, it is probably necessary to stimulate attachment by providing a suitable substrate. This could be a feeder layer of insect cells or a plastic surface covered by chitin or some related substance.

Metacyclic forms. The metacyclic forms are produced in the salivary glands of the vector. It is the stage in the life-cycle of the parasite which is infective to the mammalian host. The in-vitro production of metacyclic forms is impeded by the fact that this parasite stages does not divide. We have never observed a dividing metacyclic form in thousands of positive salivary probes examined during the past 12 years. Moreover, metacyclic forms transform in vitro within 8–10 h to slender bloodstream forms.[12] Metacyclic forms seem to be inhibited from transformation in the salivary glands by some unknown factor, most probably a constituent of the salivary secretion. If this were not the case, we should find bloodstream forms in the salivary glands. Outside the vector, metacyclic forms transform immediately to bloodstream forms and start to divide.

It has been shown that, when non-infective trypomastigotes are cultivated at 28 °C in the presence of head-salivary gland explants of *Glossina morsitans morsitans*, infective metacyclic forms are produced.[13,14] Large numbers of explants per culture are required and the percentage of metacyclic forms is normally below 0.1%. It has been demonstrated by antigenic analysis and ultrastructural studies, that these forms are true metacyclics.[15,16] Metacyclic forms can also be obtained in the presence of tsetse alimentary tract and abdominal body wall explants.[17] The proportion of infective forms in the medium was between 0.01% and 0.3%. This culture technique has enabled the production of limited numbers of metacyclic forms. However, it requires the availability of Glossina pupae and the method is rather laborious. The extremely low percentage of metacyclic forms in these cultures is perhaps explained by the low concentration of inhibitory factor outside the salivary glands. Freshly produced metacyclics which leave the glands presumably transform to bloodstream forms and eventually die because the culture conditions do not support the growth of bloodstream forms. The discovery of the nature of this 'inhibitory factor' and its isolation would allow the addition of a sufficient concentration to the culture medium to prevent transformation. This would lead to the accumulation of metacyclic forms.

Another approach has been chosen by Nyindo and co-workers using infected salivary glands with and without normal minced glands in the presence of a feeder layer of bovine embryonic spleen cells at 25 °C and 30 °C.[18] Some of these cultures produced infective trypanosomes. Since metacyclic forms do not divide, it has to be assumed that the metacyclics introduced into the culture system transformed to bloodstream stages. Bloodstream forms are able to grow in the presence of a mammalian feeder layer even at temperatures lower than 37 °C. The most interesting aspect of this culture system is the fact that it produces antigenically stable parasites at 29 °C.[19] This finding could open new ways to produce metacyclic antigens for characterization or even immunization studies.

Due to the difficulties outlined above, a simple system that produces metacyclic forms is not currently available. Further work is necessary to develop such a system. The most promising approach may be one in which epimastigotes are induced to attach to a suitable substrate as such forms are considered to be the precursors of metacyclic trypomastigotes.

Metacyclic forms do not transform into procyclic trypomastigotes when incubated with tricarboxylic acid cycle intermediates in culture medium that supports transformation from bloodstream to procyclic trypomastigotes and growth of the latter stage. This observation has been further confirmed by the finding that metacyclic forms are not able to infect tsetse flies when administered through a membrane (unpublished observations). These observations demonstrate that metacyclic forms are very much restricted to their developmental potentials. They cannot divide nor transform directly to the procyclic trypomastigote stage; the only process of which they are initially capable is transformation to the bloodstream form.

Vertebrate form. In the mammalian host, the parasite is not restricted to the bloodstream but also invades various host tissues and the lymphatic system. The term 'vertebrate forms' thus comprises bloodstream as well as extravascular trypanosomes. When vertebrate forms are grown in culture, we do not know whether the parasites develop as bloodstream or extravascular forms. In this case 'vertebrate forms' would be a correct term. However, 'bloodstream forms' is often used synonymously.

The first continuous cultivation of bloodstream forms was achieved by Hirumi and co-workers, using a bovine fibroblast culture as feeder layer in tissue-culture medium supplemented with fetal bovine serum (FSB).[20,21] The co-cultivation of trypanosomes and mammalian cells has proved to be essential for the cultivation of the vertebrate forms. Many cell lines isolated from various mammals either of the fibroblast or the epithelial cell type have now been successfully used as feeder layers for the cultivation of *T.b. brucei* and *T.b. rhodesiense*.[20–25] Primary murine bone marrow cultures also have been shown to support the continuous growth of *T.b. brucei* bloodstream forms.[26] The medium used in these culture systems is always RPMI 1640 or Eagle's Minimum Essential Medium (MEM) with Earle's salts, both media supplemented with 25 mM HEPES buffer. The addition of 20% FBS or another mammalian serum (usually heat-inactivated) is obligatory. If a fibroblast cell line is used as feeder layer, the trypanosomes grow intercellularly between the fibroblasts as well as in the supernatant medium (Figs. 1, 2). The parasites located in the feeder layer are mainly monomorphic, whereas the free trypanosomes in the medium exhibit a slight pleomorphism with usually less than 15% stumpy forms.[25] This situation closely resembles findings in infected rats: while the trypanosomes in the lymph nodes were

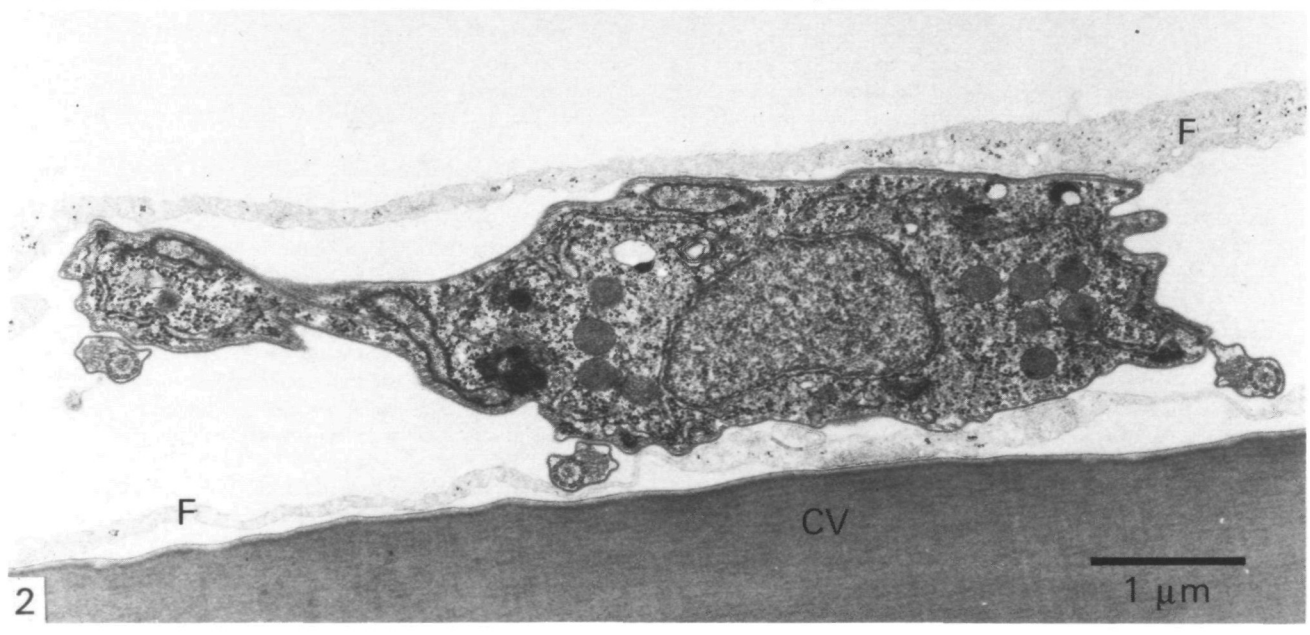

Fig. 1

Vertebrate form culture of *Trypanosoma b. brucei*. Scanning electron micrograph of a feeder layer of rabbit embryo fibroblasts with a group of trypanosomes growing over the fibroblasts. ×6400 *Courtesy of R Guggenheim and M Tanner.*

Fig. 2

Vertebrate form culture of *Trypanosoma b. brucei*. Vertebrate form growing between rabbit embryo fibroblasts (F). Bottom of the culture vessel (CV). ×21000

monomorphic and slender-like, the trypanosomes isolated from the bloodstream of the same animal were pleomorphic.[27] These observations were confirmed in a quantitative ultrastructural investigation where *T.b. brucei* grown in vitro and in vivo were compared.[28] Another important achievement has been the in-vitro cloning of bloodstream[25,29] and metacyclic forms.[25]

Vertebrate forms grown in vitro are very similar, if not identical, to the forms found in the mammalian host with regard to ultrastructure,[28] infectivity for the mammalian host and for the vector,[30] and for the successive expression of different variant surface glycoproteins resulting in antigenic variation.[31,32] The occurrence of antigenic variation in vitro is an important indication that this phenomenon is not induced by specific host antibodies. The role of the feeder cells is still unclear. Fibroblast-conditioned medium does not support growth of the parasite, but allows maintenance of bloodstream forms for up to 72h. Separation of fibroblasts and trypanosomes by membranes also results in a loss of growth of the trypanosomes.[33] These experiments indicate that there are no stable growth-stimulating factors released by the fibroblasts. Cell divisions are rather stimulated by labile, short-range factors produced by the feeder cells or by cell-contact interactions. In experiments using cell-free culture systems with fibronectins from different mammals, growth of bloodstream forms could not be obtained (unpublished observations). At present the mechanism by which the feeder cells support the growth of vertebrate forms in vitro is still unknown.

Human serum resistance, a major differentiating phenomenen between *T.b. brucei* and *T.b. rhodesiense/T.b. gambiense*, can be tested in vitro with a new and simple test.[34] The test consists of two parallel cultures; a test culture with 20% human serum and a control culture with 20% heat-inactivated horse or rabbit serum. This system is able to detect single resistant forms among a majority of sensitive forms.

For *T.b. gambiense*, the cultivation of the vertebrate form is of special importance since there is still no laboratory model with a good susceptibility for this *T.(T.) brucei* subspecies. *Trypanosoma b. gambiense* stocks from the Ivory Coast and Liberia which do not grow at all in white mice and only after a long adaptation phase in selected wild rodents such as *Microtus montanus* or *Mastomys natalensis*, can readily be grown in culture. The culture system consists of a feeder layer of *Microtus montanus* embryo fibroblasts or human embryonic fibroblasts in RPMI 1640 or MEM supplemented with 20% normal human, goat, or horse serum (all sera heat-inactivated).[25] These culture systems allow the direct isolation of stocks from human blood or cerebrospinal fluid without rodent passages, which may change the characteristics of the isolate.[35]

The relatively low trypanosome densities in all existing vertebrate form cultures (about 10^6/ml) impedes the mass production of this stage in the life cycle of *T.(T.) brucei*. The accumulation of pyruvate seems to be the main factor responsible for this growth limitation. Roller bottle or micro-carrier cultures could be a way to improve the yield of cultivated vertebrate forms. There are several applications of these vertebrate culture systems, e.g. studies of metabolism, differentiation and antigenic variation under controlled conditions, the testing of drugs, the characterization of stocks from man and animals, and the production of antigen for diagnosis and immunization studies.[36] Further research has to concentrate on the replacement of the feeder cells to obtain a cell-free system, replacement of the serum in the medium and improvement of the trypanosome density in the cultures.

Trypanosoma (N.) Congolense and *T.(D.) Vivax*

In large areas of Africa, the breeding of cattle is impeded or even impossible due to trypanosomes causing nagana. *Trypanosoma congolense* and *T. vivax* are mainly responsible for this disease which affects domestic animals such as bovines, horses, goats and sheep. Recent progress made in the cultivation of these two trypanosomes species will be briefly outlined.

The non-infective procyclic trypomastigote form of *T. congolense* can be grown in semi-defined media in the presence of Glossina cells[37,38] or in media alone.[7,39] Direct adaptation from bloodstream forms and growth of the procyclic trypomastigotes to cell densities of up to 4.5×10^7/ml can be obtained in a modified commercial medium.[39]

The first success in the in-vitro production of metacyclic forms of *T. congolense* was reported by Gray and co-workers.[40] Their cultures were initiated by placing infected Glossina proboscides beside a bovine dermal collagen explant. Procyclic, epimastigote and metacyclic forms could be found in the cultures and their infectivity for mice was demonstrated. Subpassages into medium without bovine dermal explants continued to produce metacyclic forms which were capable of infecting rabbits and produced typical local skin reactions.[41] In a modified system, the bovine dermal collagen explant could be replaced by Vitrogen.[42] This relatively simple culture system is capable of producing 10^6 and more metacyclics, although only a small proportion of the culture forms represent metacyclic trypanosomes. This is a considerable improvement over the few hundred metacyclic forms which can be obtained from one infected tsetse fly.

Until very recently, the bloodstream stage of *T. congolense* could not be grown in vitro. Limited success has been achieved by transferring infected skin into culture medium. In doing so, the infectivity of the trypanosomes was retained for up to 21 days.[43] The first continuous culture system for *T. congolense* bloodstream forms used a feeder layer of bovine endothelial cells in RPMI 1640 medium supplemented with 16% heat-inactivated adult goat serum and 4% fetal goat serum.[44] Bloodstream forms from culture were morphologically indistinguishable from trypanosomes isolated from the mammalian host. They were covered by a surface coat and proved to be infective when inoculated into mice.

Trypanosoma vivax has proved to be the most difficult of the three most important African trypanosome species to cultivate in vitro. Experiments done by Trager[45] over 25 years ago showed some limited success. Bloodstream forms brought into culture with tsetse tissue could be maintained for over one month and were capable of infecting two sheep. These experiments were repeated some years later, but this time infectivity could not be demonstrated.[46] Recently, scientists at ILRAD, Nairobi have developed a culture system to grow both procyclic and metacyclic forms of *T. vivax*.[47] Bloodstream forms from mice were introduced into a culture of bovine fibroblasts in MEM, with beads of Matrix Green Gel A at 25°C. They transformed to non-infective insect forms which attached to the beads and afterwards to the feeder cells. After 2–3 weeks, a small proportion (<1%) of metacyclic forms could be detected. This culture system represents an immense advance in the in-vitro production of metacyclic forms, since it is known that only single metacyclic forms are extruded by *T. vivax*-infected tsetse flies.

Progress in the cultivation of bloodstream forms was reported recently for a west African *T. vivax* stock in a system similar to the one used for *T.(T.) brucei*.[48] Microtus embryo fibroblasts were used as feeder cells in MEM, supplemented with 20% heat-inactivated adult goat serum. So far, east African *T. vivax* stocks have not been grown successfully in this system.

South American Trypanosomes: *Trypanosoma (S.) Cruzi*

In contrast to the African trypanosome species which are most difficult to grow through their entire life cycle, all stages of *T. cruzi*

can be grown in complex or even chemically defined media. The different forms found in the triatomid vector (amastigotes, epimastigotes, sphaeromastigotes and metacyclic trypomastigotes) can all be grown in a single culture medium. The proportion of the different forms, however, depends on the type of medium used and the culture conditions. Insect tissue culture offers additional possibilities to study the vector stages, although the presence of reduviid cells is not essential to support the growth of these forms. The cultivation of the vertebrate stages requires the co-cultivation of mammalian cells as amastigotes are obligate intracellular parasites in the vertebrate host. The high standard of tissue culture techniques facilitates the production and study of these vertebrate forms.

Insect Forms

After an infective bloodmeal, the bloodstream trypomastigotes transform in the gut of the vector to amastigote and later to epimastigote forms. Sphaeromastigote forms may arise before metacyclic trypomastigotes develop in the insect's hindgut. *T. cruzi* only develops within the alimentary canal of the vector. It is therefore not surprising that all developmental stages can be found in culture, though in varying proportions. The majority of parasites in a culture of insect forms are normally epimastigotes. These forms have been grown since the beginning of this century in various diphasic media.[49] Since that time numerous culture media have been developed to grow the insect stages. Beside complex media (e.g. refs. 50, 51) and defined media with serum supplement (e.g. refs. 52–54), there now exist chemically defined media.[55–57] These defined media are of the utmost importance in the study of the parasite's metabolism and its nutritional requirements.

Metacyclic trypomastigotes are produced in vitro with much greater ease and consistency than with species of African trypanosomes. It has been found that metacyclic forms appear at the end of the exponential growth phase, possibly due to the depletion of nutrients in the medium or accumulation of metabolites.[58] Metacyclogenesis is also favoured by special media such as LMC Medium[59] or Grace's insect tissue culture medium at a pH of 6.6.[60] In a recent investigation, metacyclogenesis was studied in two defined media, L-15 and Grace's medium.[61] It was found that serum (10% newborn calf serum), various sugars and a pH of 5.5 were required for the formation of metacyclic forms. The culture vessel used also had an influence on the production of metacyclic forms. This indicates that gaseous exchange is an important factor and requires investigation. Formation of metacyclics is also favoured by the co-cultivation with insect cells or the addition of insect extracts.[62,63] In another study, it has been observed that embryonic cells of *Triatoma infestans* promote the formation of 'staphylomastigotes' and subsequent metacyclic trypomastigotes.[64] In the absence of the insect cells, metacyclogenesis did not occur. De Isola and co-workers[65] have studied the influence of organ extracts obtained from *Triatoma infestans*. They found that the intestine and stomach of fed bugs induced a high proportion of culture forms to transform to the metacyclic stage.

It is evident from the cited research work that the production of epimastigotes or metacyclic trypomastigotes in large quantities is no major problem (Table 1). The use of continuous-flow systems (e.g. ref. 66) can improve the yield of parasites necessary for biochemical or immunological studies. The in-vitro cloning of parasites on solid medium plates represents another advance in the culture of *T. cruzi* insect forms.[67]

Vertebrate Forms

The cultivation of vertebrate forms of *T. cruzi* is more complex than that of insect forms in that the presence of mammalian cells is required. Initial successes were achieved over 40 years ago but it was not until improvements in tissue culture techniques occurred that simple and efficient methods for the propagation of vertebrate forms could be established. Work carried out up to 1978 is adequately reviewed by Pipkin,[68] Brener[69] and Dvorak.[70] The following account deals only with the more recent advances and achievements in this field.

In the mammalian host, *T. cruzi* shows two distinct forms: the intracellular amastigote form and the trypomastigote bloodstream form. The trypomastigote forms appear as slender and as broad forms. Amastigote forms multiply in various host cells before they transform intracellularly to trypomastigotes which are then released from ruptured host cells. The trypomastigote forms are non-dividing; they may infect other host cells or may be taken up by the bug vector during its bloodmeal on an infected host. Infection studies with Vero and muscle cells demonstrated that slender forms are necessary for cell infection.[71] The broad forms do not infect vertebrate cells but can develop in the triatomid vector. This situation resembles that which occurs in *brucei*-subgroup trypanosomes, in which stumpy forms show a greater infectivity for the tsetse vector than the slender bloodstream forms.

Various cell types can be used as host cells to grow *T. cruzi* vertebrate forms, e.g. chick embryo cells, bovine embryo muscle cells, Hela or Vero cells, fibroblasts, or myocardial cells (see references in ref. 72). Amastigote forms can be grown in two different ways, either in susceptible host cells or in cell-free semi-defined media.[73] Amastigotes produced in cell-free cultures are still capable of entering muscle cells and multiplying. Induction of intracellular transformation from amastigotes to trypomastigotes can be observed when FBS in the medium is replaced by horse

Table 1
Trypanosoma cruzi insect form cultures: final cell densities and morphological forms

Author	Year	Final cell density/ml	Morphological forms in culture	Medium
Cermargo[58]	1964	1.5×10^8	<25% metacyclics >75% epimastigotes	LIT
Castellani et al[50]	1967	7×10^7	40–70% metacyclics, rest epimastigotes	HIL, at pH 6.7
Pan[51]	1971	4×10^7	Mainly epimastigotes	F—29
		10^7	Mainly amastigotes	F—32
Yoshida[52]	1975	10^8	Epimastigotes, metacyclics*	Macromolecule-free medium
Wood & Sousa[53]	1976	8×10^7	≤85% metacyclics	Grace's Insect Tissue Culture Medium +0.25% *Rhodnius prolixus* extract
Azevedo et al[55]	1977	1.6×10^7	Not stated	HX 25 modified, defined medium
Avila et al[56]	1979	2×10^7	Mainly epimastigotes	Defined medium
Dusanic[59]	1980	8×10^7	>89% metacyclics	LMC
de Isola et al.[65]	1981	3×10^8	60–90% metacyclics	Modified Grace's Medium+*Triatoma infestans* digestive tract extract
Sullivan[60]	1982	5×10^7	>90% metacyclics	Grace's Insect Tissue Culture Medium
Avila et al.[57]	1983	$\sim 10^7$	Mainly epimastigotes	TMM, minimal defined medium

* Various proportions of metacyclic trypanosomes depending on trypanosome strain and medium composition

Table 2
Trypanosoma cruzi vertebrate form cultures: final cell densities and morphological forms

Author	Year	Final cell density/ml	Morphological forms in culture	Feeder cells	Medium
Pan[73]	1978	3×10^7	Amastigotes	-	F-69
Bioul-Marchand et al[72]	1980	2×10^7	Trypomastigotes	Myocardial cell line	DMEM
Sanderson et al.[80]	1980	$3 \times 10^7/25\,cm^2$	Trypomastigotes	WI 38, MRC5 human diploid cell lines	BME
		$5 \times 10^7/25\,cm^2$	Amastigotes*		
Schmatz & Murray[78]	1981	2×10^6	Only trypomastigotes on day 6, trypomastigotes & amastigotes by day 11	Myoblast cells	DMEM+M 199
Schmatz & Murray[79]	1982	$\sim 10^9/150\,cm^2$	Trypomastigotes	Irradiated myoblast cells	DMEM+M 199
Villalta & Kierszenbaum[76]	1982	3.5×10^7	Amastigotes	-	ML-15 HA
de Carvalho & de Souza[81]	1983	2.6×10^7	Only trypomastigotes	Transformed fibroblasts	MEM
		2×10^7	Only amastigotes	Macrophage-like cell line	MEM
Hudson et al[77]	1984	1.7×10^7	*Amastigotes & trypomastigotes***	Muscle derived cell line	DMEM

* Harvested by cell disruption and gradient separation
** The proportion of amastigote/trypomastigote forms varied cyclically

serum.[74] Several culture systems are described which use vertebrate cells to grow amastigotes as intracellular parasites. In a myocardial cell line in Dulbecco-modified MEM, amastigotes multiply intracellularly followed by transformation to trypomastigotes which are subsequently released into the medium.[72] Isolation and separation of amastigotes from host cells or debris can be attained by metrizamide gradient centrifugation.[75] With a metrizamide gradient it was also possible to separate infected from non-infected mastocytoma cells grown in suspension culture.[76] Ficoll gradient centrifugation can be used to separate amastigotes from trypomastigotes, as well as cell debris.[77]

The non-dividing trypomastigote bloodstream form cannot be grown directly in vitro. The only way to obtain this parasite stage is via transformation from amastigotes. This differentiation takes place in the host cell after the multiplication phase of the amastigote forms. In rat muscle cell cultures, only trypomastigotes were released after 6 days, whereas on day 11 trypomastigotes and amastigotes could be found. The parasites could be isolated by passage through a DE 52 cellulose column.[78] In another investigation, the same authors used gamma radiation to inhibit cell division of myoblast cells and thus prevent overgrowth of the host cells.[79] This treatment resulted in a more synchronous release of trypomastigotes and a higher trypanosome yield. Sanderson and co-workers[80] used two human cell lines to obtain almost pure trypomastigote forms. By varying the number of culture passages, it was even possible to select for slender or broad trypomastigotes. In addition, amastigotes could also be isolated from these cultures by disrupting the cells before trypomastigotes had appeared and separating the amastigotes by metrizamide gradient centrifugation.

Different types of host cells release different proportions of amastigote and trypomastigote forms. This difference depends on the origin and size of the host cells. It has been found that a transformed fibroblast cell line released mainly trypomastigote forms, whereas a macrophage-like cell line mainly released amastigotes.[81] It is a great advantage to have cell lines which produce only amastigotes or only trypomastigotes. For the isolation and purification of the parasites centrifugation can be applied using Ficoll or metrizamide gradients or by passage through DEAE cellulose. However, it has been found that the latter method decreases the infectivity of the parasites.[82]

A decrease of virulence was found in trypomastigotes grown in co-cultivation with Vero or fish cells at 30°C. Mice infected with these attenuated forms became immune to challenge. Parasites grown at 37°C did not show this loss of virulence.[83] For biochemical and immunological investigations large quantities of parasites are required. With the available tissue culture techniques, it is possible to produce sufficient quantities of amastigotes or trypomastigotes to conduct such studies (Table 2). The expected yield of parasites can be further increased by the use of roller bottles[84] or microcarrier cell culture which increases the surface area of adherent cells available to the parasites.

Interaction with Macrophages

One major application of culture methods for *T. cruzi* is the study of interactions of parasite and macrophage in vitro. Epimastigotes from axenic culture, as well as trypomastigotes from blood or culture, are taken up by macrophages. The highest infection rate in macrophages has been obtained with tissue culture derived trypomastigotes (60%), the lowest with bloodstream trypomastigotes (10%).[85] Entry of the parasite into the host cell is achieved by endocytosis, as demonstrated by the inhibitory effect of Cytochalasin B.[86] The parasite strains themselves have different affinities for the macrophages, e.g. Y strain always gives higher infection rates in mouse macrophages than CL strain.[85] It could be shown that activated macrophages and macrophages pretreated with specific antibodies, achieved a better uptake of *T. cruzi* parasites than normal macrophages.[87] Another important parameter that influences the fate of the parasite in the host cell is the ratio of parasites to macrophages. A high ratio may lead to the destruction of the host cells whereas a lower parasite ratio can be controlled by the macrophage. Internal parasites within host cells located outside cytoplasmic vacuoles survive while the forms found in phagosomes were destroyed.[88] The interaction of parasite and macrophages has been studied extensively using electron microscopy (e.g. refs. 89, 90).

Conclusions

Most of the parasite stages of the life-cycle of the three most important African trypanosome species *T.(T.) brucei*, *T. congolense* and *T. vivax* can today be grown in vitro. However, only a few systems exist which are simple and inexpensive. The only stages which can easily be grown in semi-defined media to high parasite densities are the procyclic trypomastigotes. Metacyclic forms are much more difficult to produce in that they require cells/explants from insect or mammalian hosts. Metacyclic trypanosomes are always produced in mixed cultures from procyclic forms and usually represent less than 1% of the total number of cells present. The vertebrate stages are easier to produce than the metacyclic forms but require a mammalian cell feeder layer. For mass cultivation of this parasite stage, the trypanosome density has to be significantly increased. The most promising method of achieving this goal would seem to be the removal of the parasite's metabolites from the culture medium.

When growing parasites in vitro, special attention has to be paid to retaining the parasite's natural characteristics. By selecting feeder cells and serum supplement from a susceptible host, a 'natural' culture system can be obtained. In such a culture system the trypanosomes are more likely to grow and react in a comparable way to that in the vertebrate host. This is especially important for in-vitro studies of metabolism and the effect of drugs. Due to the trypanosome's ability to adapt to environmental (culture) conditions, parasites can be produced which possess a divergent metabolism as compared to the parasites which grow in their natural hosts. Long-term maintenance of trypanosomes in culture may also lead to altered parasite populations which can no longer be considered to represent the parasites as they occur in the field. The constant selection in culture can easily change the characteristics of the parasite population.

Isolation of parasites directly from the insect vector into insect stage culture medium, without passage through a vertebrate host, offers a way to obtain this parasite stage with the full potential it possesses in the vector. In general, it is important for most studies to use recently established cultures. Parasite populations which have been in culture for several years may have lost some of the characteristic features they normally possess in their natural hosts.

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